Antimalarial drug resistance markers in HIV-positive and HIV-negative adults with asymptomatic malaria infections in Port Harcourt, Nigeria.

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Original Article
Abstract

Background: In Nigeria, indiscriminate use of antimalarial drugs may contribute to the threat of drug resistance, but has not been evaluated among people living with HIV.

Methods: HIV-positive adults attending a university hospital HIV clinic and HIV-negative adult volunteers from the university hospital community with a positive blood film were treated with artemether-lumefantrine. Parasite DNA from before and after treatment was PCR-amplified to identify molecular markers of drug susceptibility.

Results: The pfcr76T genotype was prevalent among both HIV-positive and HIV-negative participants (78.6%, 68.2%). Three new mutations in the pfmdr1 gene, F73S, S97L and G165R, and the uncommon pfldps S436F variant were detected, whereas pfldpsK540E and pfldfwr164L were absent. The A437G allele of pfldps predominated (62/66, 94%). The I431 V mutation was found in 19 out of 66 pre-treatment pfldps sequences (28.8%). The pfmdr1 86N allele was significantly more common at day 3 post-treatment than at baseline (OR 8.77; 95% CI 1.21 – 380).

Conclusions: We found evidence of continued chloroquine use among HIV-positive individuals. Selection for pfmdr186N after AL treatment was observed, indicating a possible threat to antimalarial efficacy in the study area. The complexity of pfldps haplotypes emphasises the need for careful monitoring of anti-folate susceptibility in Nigeria.

199 words

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Introduction

Despite improved control measures in many countries, malaria remains a leading cause of morbidity and mortality in sub-Saharan Africa, especially in pregnant women and children. Co-infection with HIV, which is equally endemic in the region, increases the threat from both diseases, because of the geographic overlap between them\(^1\). Malaria and HIV/AIDS are both diseases of poverty, which work together to deepen poverty in the communities where they co-exist; co-infection is thought to contribute to 3 million additional malaria cases, higher malaria parasite densities in children and a 5% greater mortality\(^2,3\). HIV infection may also change pharmacokinetics, effectiveness of antimalarial use and treatment-seeking patterns, leading to more frequent contact with drug vendors, but this has not been adequately assessed in endemic regions\(^4,5\).

The employment of molecular markers has proved very effective for surveillance of antimalarial drug resistance\(^6,8\), but can also reflect community patterns of drug use and inform drug policy\(^9\)(Mugittu et al., 2004). The report of the reemergence of chloroquine-sensitive parasites in areas where it had been withdrawn illustrates the benefit of this approach\(^10\). Molecular markers have been utilized as tools for surveillance of resistance in *Plasmodium falciparum* populations in Africa\(^6,11\) provide additional data that compliment clinical observations of the *in vivo* efficacy of a drug\(^12,14\), and have been instrumental to policy-making with regards to control of malaria epidemics\(^9\). These markers also serve as tools for monitoring parasite drug susceptibility following changes in treatment policy leading to altered drug pressure on human infections\(^10,15\). Therefore, the continued presence of such markers in a population after changes in antimalarial policy is likely to be a useful indicator of persisting unregulated use of outdated regimens, a behaviour that may be more common among people living with HIV.

In this study, we seek evidence of continued use of older antimalarials among asymptomatic HIV-positive (HIV\(^+\)) and HIV-negative (HIV\(^-)\) individuals in Port Harcourt, Nigeria, by testing the hypothesis that higher carriage of resistant parasites among the HIV\(^+\) group is an indication of greater use of therapies obtained from the informal sector. We report genotypes of the loci *pfmdr1* and *pfcr* for chloroquine, and *pfdhfr* and *pfdhps* for sulfadoxine-pyrimethamine (SP), before and after treatment with artemether-lumefantrine (AL). We also test for evidence of selection on the *pfmdr1* locus, as previously reported in AL-treated symptomatic malaria patients\(^13,14,16\).
Materials and Methods

Blood sample collection

A total of 459 finger-prick blood samples were collected from asymptomatic HIV+ and HIV- adult subjects, volunteering at either University of Port Harcourt Teaching Hospital or Braithwaite Memorial Specialist Hospital in Port Harcourt, Nigeria between October, 2010 and September, 2011. HIV+ individuals were contacted at outpatient clinics in both hospitals during routine attendance for CD4 T-cell monitoring and dispensing of anti-retroviral medication. HIV- individuals were recruited as volunteers from among the hospital and university communities. Participants were offered a free malaria diagnostic test and ACT treatment if positive, services that usually incur a cost. All participants provided written informed consent, completed questionnaires and were initially screened for *Plasmodium falciparum* parasites as previously described. Patients came from all over the Niger Delta region since these are the two largest hospitals in Rivers State. Screened subjects found to be parasitaemic by microscopy were treated with artemether-lumefantrine, and asked to return for follow-up visits on day 3, 7 and 28. Approximately 50 µl of blood was spotted directly from the finger onto glass fibre printed filtermat (WALLAC Oy., Turku, Finland) on days 0, 3, and 28 for parasite DNA extraction. Filter papers were allowed to air-dry and then stored in individual pouches with desiccant and transported to the London School of Hygiene and Tropical Medicine (LSHTM) for PCR analysis. A pharmacokinetic study of a subset of the participants has been described previously.

DNA extraction and molecular marker identification

DNA was extracted from blood spots on filter papers to yield approximately 100 µl using a modified Chelex extraction method as previously described. Samples collected on day 0 were analysed for polymorphisms in the chloroquine resistance transporter (pfcrtr) at codons 72-76 by qPCR as previously described. For pfcrtr genotyping in low density parasite samples, extracted DNA first amplified using primers CRTP1 and CRTP2 of Djimde et al., as a nest 1 amplification, before analysis by qPCR. Direct sequencing of amplicons of the multidrug resistance gene (pfmdr1), dihydopteroate synthetase (pfdhps) and dihydrofolate reductase (pfdhfr) was performed as previously described.

Statistical analysis

All data collected were entered into spread sheets and analysed in STATA version 16.1 (Stata Corp., Madison WI). Statistical associations between binary variables were tested in 2x2 tables using the chi-sq distribution.

Ethics approval
The study was approved by the Ethics Committee of the University of Teaching Hospital, Port Harcourt, Braithwaite Memorial Hospital Ethics Committee, Port Harcourt and by the LSHTM Ethics Committee (Ref # 5817, 2010).
Results

A total of 62 HIV+ individuals and 80 HIV- individuals were positive for *P. falciparum* DNA by at least one PCR assay at day 0, of which 17 and 20, respectively, were previously included in our pharmacokinetic study. Filter paper blood samples collected at day 3 were available for 48 and 45 participants, with 24 of these positive by *pfmdr1* PCR, indicating either persisting parasitaemia or the presence of *P. falciparum* gametocytes. 28 of these 93 individuals were PCR positive at day 28 (Fig. 1). All participants were asymptomatic at enrolment.

**Pfcrt polymorphisms**

Only a proportion of PCR-positive blood samples had sufficient parasite density to generate enough DNA for successful marker sequencing. Among the day 0 pre-treatment samples, alleles of the *pfcrt* codons 72-76 were successfully analysed for 72 samples on day 0. Analysis of the data showed that the mutant *pfcrt*76T is predominant (72.6%) in the study area. The wild-type *pfcrt*76K (chloroquine sensitive) allele was more common among HIV-negative persons than HIV-positive, but this difference was not significant (OR: 2.5; 95% CI: 0.82-7.95; P= 0.072). The two *pfcrt* haplotypes at codons 72-76 that dominate in Africa were both present: the wild type CVMNK and the mutant type CVIET (Table 1). We did not detect the haplotype SVMNT, which has been associated with AQ resistance. Analyses of the day 28 post-treatment samples yielded only 5 interpretable genotypes from each group, 3 and 4 of which were CVIET among HIV+ and HIV- participants, respectively. We were not able to generate meaningful genotypes for *pfcrt* in the day 3 samples.

**Pfmdr1 polymorphisms**

A total of 68, 66 and 79 out of these samples were successfully amplified by *pfmdr1* PCR and sequences obtained for codons 86, 184 and 1246, respectively. Only wild type alleles were found at codons 1034 and 1042. Molecular analysis of the codon 86 sequences showed a predominance of the wild type asparagine (86N) allele with a 75% (51/68) prevalence compared to 13.2% (9/68) for tyrosine (86Y) in a pure form and 12% (8/68) for mixtures or other alleles (including F) on day 0 (Table 2). Post-treatment samples collected during follow-up on day 3 were analysed for mutations at the same loci. The genotypes showed a more extreme pattern, with 96.9% (31/32) 86N and the 86Y occurring in only a single individual (3.1%).

Baseline distribution of polymorphisms at *pfmdr1* codon 184 showed a high prevalence of the wild-type allele Y184 alone or mixed (63.6%, 42/66; Table 2), although the mutant allele Y184F was more prevalent among HIV- individuals (18/30, 60.0%) than among those that were HIV+ (13/36, 36.1%; OR 2.65; 95% CI 0.88 - 8.12; P = 0.053). Predominance of the wild-type was maintained at day 3 after treatment, combining both HIV status groups. At codon 1246, the Y allele associated with aminoquinoline resistance was present at 10%
prevalence or less at all timepoints (Table 2). Among the 45 pre-treatment *P. falciparum* isolates with only a single allele at each variant position, 6 different *pfmdr1* haplotypes were observed either in single or mixed forms (Fig. 1). The most prevalent haplotype among pre-treatment isolates was NYD (27 isolates, 60%) followed by NFD (9 isolates, 20%). At day 3, 25 unambiguous *pfmdr1* haplotypes could be assigned, including 16 NYD (64%), six NFD (24%) and one each of NFY, NYY and YYD. Evidence was found for selection by AL treatment favouring the *mdr*86N wild type, which increased in prevalence from 77.9% at day 0 to 96.9% at day 3 (OR 8.77; 95% CI=1.2-380; P=0.016). Sub-group analysis showed such selection also occurred within the HIV+ group but this was not significant with an OR of 3.05 (CI=0.8-12.85; P=0.063).

**New polymorphisms in pfmdr1**

Three *pfmdr1* polymorphisms (F73S; S97L; G165R) which have not been previously reported were identified in three of our day 0 study samples, all from HIV individuals. We also found six occurrences of the N86F mutation previously reported in Swaziland and Afghanistan.\(^{17, 22, 23}\)

**Pfdhps polymorphisms**

PCR amplification of the *pfdhps* gene was successful for 67 isolates on day 0. As selection on this locus is not expected under AL treatment, post-treatment isolates were not evaluated. Molecular analysis of the sequences showed a predominance of the mutant A437G (62/66 (94%). The mutation at codon 431 (I431V), first described in 2009 and subsequently shown to be emerging across Nigeria,\(^{21, 24}\) was found in 19 samples (28.79%) and so was already circulating quite commonly in the Port Harcourt area in 2010-11. I431V always occurred with the A437G mutation, and evidence was found of strong linkage with the 581G mutation (OR 9.75; 95% CI 2.05 – 51.2; P = 0.0004) and the 613S mutation (OR 5.63; 95% CI 1.29 – 24.7; P = 0.005). The *pfdhps* variant S436F, rarely reported in Nigeria and of unknown impact on SP efficacy,\(^{24}\) was encoded in a single individual at day 0. The crucial K540E variant, associated with high level SP resistance, was not found, as all isolates encoded the wild type residue at this position. No difference was observed in the prevalence of mutant alleles of the *pfdhps* gene between the HIV+ individuals and the HIV- control group. Unambiguous haplotypes of the *pfdhps* gene were assembled for 53 isolates (Fig. 2). The most common *pfdhps* haplotype was ISGKAA (55.0%) followed by VAGKGS (15.0%) and VAGKAA (11.7%), with the wild-type sulfadoxine-susceptible haplotype ISAKAA occurring in only 3 individuals (5.0%).

**Pfdhfr polymorphisms**

Forty-eight full gene fragments encompassing the loci of interest (50, 51, 59, 108 and 164) were successfully amplified on the *pfdhfr* gene. Molecular analysis of the sequences showed a predominance of the triple mutant (N51I, C59R, S108N, I164) with a 96% prevalence. There was an absence of the *pfdhfr* I164L mutation
associated with high-level SP resistance. No single isolate carried the pure wild type haplotype NCSI, suggesting that this parasite population continues to experience anti-folate drug pressure. As for the \textit{pfdhps} gene, there was no difference in the prevalence of mutant alleles of the \textit{pfdhfr} gene between the HIV-positive individuals and the HIV-negative control group.
Discussion

In this paper we present the prevalence of *P. falciparum* gene variants associated with antimalarial drug susceptibility among HIV+ and HIV- individuals with PCR-confirmed asymptomatic malaria infections in Port Harcourt, Rivers State, Nigeria. Participants were treated with AL and followed up, with a proportion showing evidence of persistent parasitaemia at day 3 and/or day 28. This is consistent with previous studies of ACT treatment in asymptomatic individuals in high transmission African settings. An important observation from our study is the strong selection for the *pfmdr1* 86N allele among day 3 study isolates, which was also evident in the HIV-positive arm alone. This allele has been suggested as a first step to lumefantrine tolerance and its selection has been associated with AL treatment failure or slow clearance in a number of African studies. This therefore calls for close monitoring of *pfmdr1* genotypes in order to monitor the efficacy of AL in the area. The NFD haplotype, also associated with slow clearance to AL, was common in the overall population (Fig. 1), suggesting studies of AL effectiveness are needed. The NFD haplotype has previously been reported in South-Western Nigeria among children with uncomplicated malaria.

Our results also show an unexpected presence of *pfmdr1* N86F in six pre-treatment parasite isolates, and these are all from the HIV-negative people. This polymorphism was reported in clinical isolates from Afghanistan and in samples from Swaziland. The significance of this genotype for quinoline or AL susceptibility is not clear but suggests the need for investigations of phenotype in the case of new mutations. The most likely explanation for this is that the withdrawal of CQ may occasionally result in the change from the resistant mutant form tyrosine (Y) to phenylalanine (F), as this requires only a single base-pair change whereas two base changes are required from 86N.

Despite the withdrawal of CQ as first-line drug for malaria treatment, there was a high prevalence of *pfcr76T* implying selection as a result of continuous use of CQ in the environment. The high prevalence of this allele corroborates the study in Lagos, South-west Nigeria indicating a 96% prevalence of *pfcr76T*. This high prevalence is not surprising since CQ is still obtainable in most parts of Nigeria. The higher prevalence of *pfcr76T* in the HIV-positive group (non-significant) suggests that parasites in HIV+ hosts are more likely to encounter CQ drug pressure. The 76T allele seems to exhibit an inverse relationship with AL efficacy because it is selected against by this ACT in Africa, while the wild type allele *pfcr76K* has been implicated in recurrent infections following AL treatment. However, the generalisability of our findings is reduced due to two weaknesses in our study. Firstly, recruitment was amongst people with access to either of two large teaching hospitals and not representative of other communities. Secondly, our sample size was relatively small. Larger studies in HIV/malaria co-infected individuals from wider community samples are required to verify our preliminary results reported here. In addition, we cannot rule out that ARV drugs used by the
majority of our HIV+ population also select for different parasite genotypes, as pharmacokinetic interactions with antimalarial drugs are known to occur. \(^5\)

In addition to \textit{pfmdr1} and \textit{pfcrt} genotypes, we report resistance associated variants in \textit{pfdhfr} and \textit{pfdhps}, loci which encode the target enzymes of the anti-folate drugs pyrimethamine and sulfadoxine, which are administered in the combination SP. Although no longer recommended as front-line therapy, SP remains in use for chemoprevention in pregnant women, children under 5 and infants.\(^3\) The triple mutant of \textit{pfdhfr}, encoding the haplotype IRN at codons 51, 59 and 108, comprised 96% of the population. However, the \textit{pfdhps} displayed a much more complex pattern of diversity in this relatively small sample of parasite isolates (Fig. 2). Of particular interest is the finding that, in 2010-11 in Port Harcourt, the I431V mutation was already quite prevalent, and demonstrated strong linkage with the 437G, 581G and 613S mutations, as we have previously observed.\(^2\),\(^24\) This may reflect selection not only by sulfadoxine, but also by the closely related antibiotic sulfamethoxazole, a component of the antimicrobial combination co-trimoxazole. This regimen is commonly used throughout Africa, including for prevention of bacterial infections in HIV+ individuals receiving ARV chemotherapy, as was the case for the majority of our HIV+ participants. \(^4\)

**Conclusions**

The apparent selection for \textit{mdr86N} on day 3 after treatment with AL for PCR asymptomatic individuals suggests a threat to the efficacy of AL in the study area. There is therefore urgent need for active surveillance studies to be carried out that include molecular markers of ACT susceptibility. Our data confirm the emergence of complex haplotypes of \textit{pfdhps} in the Niger Delta, where there is a paucity of published data on malaria studies. This study is the first to compare the presence of markers among HIV-infected and HIV-negative people in this region of Nigeria and provides preliminary evidence of differential patterns of antimalarial drug use between these two groups.
Authors’ contributions

ICN, CAN and CJS designed the study protocol; ICN carried out the clinic liaison and sample collection; ICN and CAN prepared IRB submissions; CAN provided academic support and supervision; ICN, MCO and KBB extracted DNA from bloodspots, and performed the qPCR and sequencing studies; ICN, KBB and CJS analysed and interpreted these data. ICN and CJS drafted the manuscript; all authors read and approved the manuscript. CJS is guarantor of the paper.

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Competing interests

The authors declare no competing interests.

Ethical approval

The study was approved by the Ethics Committee of the University of Teaching Hospital, Port Harcourt, Braithwaite Memorial Hospital Ethics Committee, Port Harcourt and LSHTM Ethics Committee (Ref # 5817, 2010). Our procedures followed the Helsinki Declaration (2008 amendment) and written consent was obtained from all participants.

Data Availability Statement

The data underlying this article will be deposited with the Worldwide Antimalarial Resistance Network (WWARN) in disaggregated form, upon acceptance for publication.
References


Figure 1.  Sample flow diagram for PCR-positive *P. falciparum* infections

62 and 80 PCR-positive isolates were obtained at day 0 from among 145 and 314 individuals in the HIV+ and HIV- groups, respectively, who participated in malaria screening. All PCR-positive participants had received AL treatment at day 0. Day 3 and Day 28 filter papers were not available for all 142 individuals. The 3rd row indicates day 3 PCR results for evaluable samples. The 4th row indicates day 28 PCR results for those individuals also sampled at day 3. Individuals sampled at day 28, but not at day 3, are omitted (1 and 7, respectively, in the HIV+ and HIV- groups).

Figure 2.  Relative frequency of *pfmdr1* haplotypes in 45 evaluable day 0 *P. falciparum* isolates

Isolates from day 0 displaying a single allele at each of *pfmdr1* codons 86, 184 and 1246 are represented as haplotypes across these three positions. The NYD haplotype is the fully chloroquine-sensitive wild-type. The 1246Y allele was present at low frequency in the population.

Figure 3.  Relative frequency of *pfdhps* haplotypes in 53 evaluable day 0 *P. falciparum* isolates

Isolates from day 0 displaying a single allele at each of *pfdhps* codons 431, 436, 437, 540, 581 and 613 are represented as haplotypes across these six positions. The ISAKAA haplotype is the fully sulfadoxine-sensitive wild-type.
Fig. 1

- **Screening**
  - HIV +: 145
  - HIV -: 314

- **Day 0 PCR positive**
  - HIV +: 62
  - HIV -: 80

- **AL treatment**
  - HIV +: 16 pos, 32 neg
  - HIV -: 8 pos, 37 neg

- **Day 3 PCR**
  - HIV +: 7 pos from 14
  - HIV -: 13 pos from 28

- **Day 28 PCR**
  - HIV +: 1 pos from 8
  - HIV -: 7 pos from 30
Figure 2

Haplotypes of *pfmdr1* at day 0

Figure 3

Haplotypes of *pfdhps* at day 0